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In-vivo and *ex-vivo* inhibition of intestinal glucose uptake: A scope for antihyperglycemia

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ABSTRACT

Objective: To study hypoglycemic effect of *Phyllanthus amarus (P. amarus)* leaf extract and its glucose uptake inhibition effect in rat small intestine *ex-vivo* and *in vivo* models. **Methods:** Hypoglycemic studies were carried out in glucose loaded and streptozotocin (STZ) induced diabetic *albino* rats. Blood glucose levels were estimated at I, III and IV hour time intervals after administration of aqueous leaf extract of *P. amarus*. The study on the effect of plant extract on intestinal glucose absorption in rat was carried out using everted gut sacs. **Results:** The blood glucose levels were significantly depleted in the animals administered with aqueous leaf extract of *P. amarus* (250 mg/kg body weight). Incubation of the rat everted intestinal sacs with the aqueous leaf extract of *P. amarus* resulted in the inhibition of glucose transport across the intestinal membrane. **Conclusions:** The kinetic studies on the glucose transport inhibition across the intestinal membrane by the plant extract was a non competitive type of inhibition of the intestinal glucose transporter protein (GLUT2 and SGLT1) revealing the probable mechanism of hypoglycaemic effect of the aqueous leaf extract of *P. amarus*.

1. Introduction

In drug discovery and development, medicinal herbs have consistently been considered the leading source of pharmaceuticals, employed in the treatment of various human diseases due to their high chemical diversity and broad biological functionality[1].

Diabetes mellitus and obesity remains the most common disorders of carbohydrate metabolism. Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycaemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both[2].

It affected about 171 million people worldwide in 2000 and the number is projected to increase to at least 366 million by 2030[3]. One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycaemia. This is done by inhibiting the glucose absorption at the small intestine[4-6].

Plants continue to play an important role in the treatment of diabetes. The increase in demand in industrially-developed countries to use alternative approaches to treat diabetes, such as plant-based medicines, is also due to the side effects associated with the use of insulin and oral hypoglycaemic agents[7]. More than 400 plants are being used in different forms to treat diabetes[8]. There are more than 200 pure compounds from plant sources that have been reported to show blood glucose lowering activity[7]. The wide variety of chemicals classes indicates that a variety of mechanisms of action are likely to be involved in lowering blood glucose levels. In this study, *P. amarus Schum* was selected on the basis of their use in traditional medicines throughout Southeast Asia and experimentally determined its hypoglycaemic activity *in-vivo*.

Extracts of this species was tested for its inhibition effect on glucose transport across the rat gut *in-vivo* and *ex-vivo* (everted gut sacs). *P. amarus Schum* (Family:

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Euphorbiaceae) is a widely distributed small erect, tropical annual herbal shrub whose stem has green capsule.

The seeds are longitudinally rogues[8]. It is found in parts of South India, Florida, Mexico and throughout Southern America[9]. *P. amarus* locally known as Keela Nelli. *P. amarus* has been reported to have several pharmacological activities.

Some of the other properties of this plant might exert beneficial effects during diabetes in addition to the glucose absorption inhibition reported in the present study. Antioxidant activity[10], lipid lowering activity[11] and anti-inflammatory effect[12], may all play a part in the treatment of the various pathogenic effects of diabetes.

The present study is undertaken to estimate the glucose uptake inhibition activity of *P. amarus* aqueous leaf extract across the rat gut *in-vivo* and everted rat gut sacs *ex-vivo*.

2. Materials and methods

2.1. Animals

Male *albino* rats of Wistar strain weighing around 160-180 g were procured from Tamilnadu Veterinary and Animal Sciences University, Chennai. The animals were kept in polypropylene cages (three in each cage) at an ambient temperature of (25±2) ℃ and 55%-65% relative humidity. A 12/12 h light and dark schedule was maintained in the animal house and animals were acclimatized to the laboratory conditions, fed with commercially available rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water.

2.2. Plant material

The *P. amarus* (Family: *Euphorbiaceae*) leaves were collected from Vellore District of Tamilnadu, India during August to October, 2009. The leaves were cleaned, shade dried, authenticated and a voucher specimen (No: VCV/43/2009) is kept at the Department of Botany, Voorhees College, Vellore - 632 001, Tamilnadu, India.

2.3. Plant extracts preparation

Fresh leaves were washed with distilled water and shade dried. The shade dried leaves were powdered in an electrical blender and stored at 5 ℃ until further use. The powdered leaves were taken 100 g each and mixed with 200 mL of distilled water and were stirred magnetically at room temperature. The residue was removed by filtration and the aqueous extracts were used for experiments.

2.4. Hypoglycemic studies

Hypoglycemic studies were conducted in male *albino* rats as described earlier[13,14]. The studies were conducted in two animal models. Glucose loaded rat model and diabetic rat models.

2.5. Glucose loaded model

Plant extracts were fed to the fasted rats. After half an hour interval 10% glucose solution (1.5 g/kg body weight) was fed by oral administration and blood glucose samples were collected for glucose estimation at I, III and IV hours of interval.

2.6. Diabetic model

The animals were subjected to fasting overnight and diabetes was induced by a single intra peritoneal injection of freshly prepared solution of streptozotocin (Sigma, USA) 35 mg/kg body weight in 0.1 M cold citrate buffer of pH 4.5. Streptozotocin (STZ) was used to induce diabetes in animals by various authors[15-17].

The animals were allowed to drink 5% glucose solution overnight to bring-on the drug induced hypoglycemia, control rats were administered with citrate buffer alone. The animals were considered diabetic if the blood glucose values were above 250 mg/dL on the third day after STZ injection.

2.7. Ex-vivo everted gut sac preparation to study glucose absorption inhibition

The everted rat gut sacs were prepared according to the method described by Wilson and Wiseman[18].

2.8. Experimental design and surgical procedure

Adult male Swiss *albino* rats weighing 160-180 g were housed at room temperature and were used in this experiment. Before each experiment, the animals were starved for twelve hours but allowed for tap water use. Rats were sacrificed by cervical dislocation. The abdomen was opened by a midline incision. The entire small intestine was removed quickly by cutting across the upper end of the duodenum and the lower end of the ileum, and by stripping the mesentery manually. The small intestine was then washed with normal saline solution (0.9% w/v NaCl) using a syringe equipped with blunt end.

2.9. Preparation of everted gut sacs

Intestinal segments (10±2) cm were everted and the sacs were filled with 0.5 mL of the incubation medium (serosal fluid) and were placed in 25 mL Erlenmeyer flasks with 5 mL of the same medium (mucosal fluid). After oxygenation of the flasks with 100% O_2 for 1 min, they were tightly stoppered and kept in a shaker (90-110 oscillations/min) for 1 h at room temperature. The incubation medium was Krebs-Henseleit Bicarbonate buffer (KHB). The composition of the buffer was (mM/L): $NaHCO_3$ 25; NaCl 118; KCl 4.7; $MgSO_4$ 1.2; $CaCl_2$ 1.2; and Na_2EDTA 9.7 mg/L. Glucose (5.5-8.5 mM) was added to the medium just before the start of appropriate experiment.

2.10. Evaluation of intestinal glucose uptake under the influence of plant extracts.

For studying the effect of the plant extract on glucose (substrate) uptake, glucose was added into mucosal compartment fluid just before the start of the experiment. The aqueous leaf extract was also added in the same compartment (3.62 mg/mL). At the end of the incubation period (1 h), the sacs were removed from the flask and these sacs were emptied and the serosal fluid from the sacs was used for the estimation of glucose. Similar estimations were also performed on samples of mucosal fluid in the flasks. Glucose concentrations were measured using a commercially available glucose oxidase kit (Lifechem TM -Glucose-LR). The loss of glucose from the mucosal fluid assumed to represent the glucose taken up by the intestine, and the rise in glucose in serosal fluid, the glucose released. The difference is attributable to the glucose retained in the tissue. The amount of glucose transported from the mucosal compartment was characterized as "Uptake" while the serosal gain of the substances is treated as "release". Uptake and release of glucose was expressed as PM/g tissue wet weight/h.

2.11. Kinetic study on ex-vivo glucose uptake

A kinetic study was conducted to understand the transport/inhibition of glucose across intestinal membrane. In terms of enzyme kinetics, the amount of glucose transported/hour was analogue to the velocity of transfer, in other words, to the concentration difference of the glucose between compartments at the beginning and end of an experiment[5].

The Michaelis-Menten constant (K_m), which is the affinity of the transferring enzyme (glucose transporter) for the substrate (glucose). The maximal velocity (V_{max}), which is the rate of transfer reaction in the presence as well as in the absence of the plant extracts were determined from the differences of the uptake and release values using Michelis-Menten and the Lineweaver-Burk Plots in Microsoft excel. Comparison of difference between the control and experimental groups were examined using one-way analysis of variance (ANOVA). In each series of experiments, control everted gut sacs derived from the same rat in a buffer containing no substrate(glucose) were run in parallel. The controls were run either with or without plant extract and results were corrected accordingly.

2.12. Statistical analysis

The results were expressed in mean + standard deviation. Statistical analysis was carried out by using one way ANOVA as in standard statistical software package of social science (SPSS) version 12.

3. Results

Table 1.
Hypoglycemic effect of aqueous leaf extract of traditional medicinal plant P. amarus: a) Glucose loaded model; b) Streptozotocin (STZ) induced diabetic model-Levels of blood glucose at I, III and IV hour of intervals.

a) Glucose loaded model

Normal (*n*)	Control (C)	Experimental groups		
		I h	III h	IV h
78.00±1.09	110.00±1.89	101.00±2.77*	74.00±8.98*	65.00±7.62*

b) STZ induced diabetic model

Normal (*n*)	Diabetic control (DC)	Experimental groups		
		I h	III h	IV h
78.00±2.09	389.00±5.69	290.00±10.48*	257.00±5.09*	175.00±9.45*

Data are expressed as Mean ± SD of 6 individual observations. Values are expressed as mean ± SD of six experiments. *P<0.000 1.

Table 2.
Effects of *P. amarus* aqueous leaf extract on the uptake of the varying concentrations of glucose by everted gut sacs of rats.

Glucose concentration (mM)	Uptake (μ mol/g tissue wet wt/h) Control (*n*=6)	Uptake (μ mol/g tissue wet medium he *P. amarus* (3.62 mg/mL) (*n*=6)
5.5	45.45 ± 4.42	37.03 ± 2.42*
6.5	57.14 ± 2.60	40.00 ± 1.37*
7.5	68.96 ± 3.23	47.00 ± 1.59*
8.5	100.00 ± 2.53	68.96 ± 2.51*

The gut sacs were incubated in Kerbs-henseleit buffer (pH=7.4) at 37 ℃. Values are expressed as mean ± SD of six experiments. *P<0.000 1. *n*=number of sacs used.

Table 3.
Effect of *P. amarus* aqueous leaf extract: Kinetic parameters of the transport of D-glucose at different concentrations (5.5-8.5) across the rat everted gut sacs.

Experiments	V_{max} (mM/h)	K_m (mM)
Control (*n*=6)	0.125	20.83
P. amarus (3.62 mg/mL) (*n*=6)	0.071	20.53

Km and V_{max} values were obtained from Line weaver-Burk Plot.
Km: Michaelis-Menton constant, which is the affinity of the transferring enzyme for the substrate. V_{max}: Maximal velocity, which is the rate of transfer reaction, in the presence as well as in the absence of *P. amarus* aqueous leaf extract determined from the differences of uptake and release values using Michaelis-Menten and Lineweaver-Burk Plots in Microsoft Excel.

A significant elevation of blood glucose level was recorded in the glucose loaded group when compared to that of normal. The results revealed a significant blood glucose lowering effect in the *P. amarus* leaf extract (250 mg/kg body weight) administered group at I hour, III hour and IV hour intervals when compared with that of the control (Table 1a). After 48 h of the injection of STZ to the normal rats diabetes was evidenced.

The blood glucose level was significantly elevated in the STZ injected rats when compared to that of normal (placebo) and therefore considered as diabetic animals (Control). The plant extract administered diabetic animals recorded a significant blood glucose lowering effect at I h, III and IV h intervals (Table 1b). Incubation of the rat everted gut sacs with the aqueous extracts of *P. amarus* at concentration 3.62 mg/mL resulted in the inhibition of transport of glucose. The glucose transport inhibition was significant at varied concentrations i.e. 5.5, 6.5, 7.5 and 8.5 mM/L in the incubation medium (Table 2). The pattern of glucose uptake *ex-vivo* in various experimental protocols was analyzed using Michelis-Menten and Lineweaver-Burk Plots.

The Km (Michaelis-Menton constant, which is the affinity of the transferring enzyme for the substrate). V_{max} (Maximal velocity, which is the rate of transfer reaction, in the presence as well as in the absence of aqueous leaf extract was determined from the differences of uptake and release values using Michaelis-Menten and Lineweaver-Burk Plots in Microsoft Excel) as single entities.

The value of V_{max} was significantly depleted in the entire experimental samples incubated along with the plant extract when compared with the control. However the Km remained constant throughout the study (Table 3).

4. Discussion

The most challenging goal in the management of diabetes mellitus is to achieve blood glucose level as close to normal as possible. The importance of postprandial glucose control in the development of diabetic complications is widely recognized based on the direct stimulation of endothelial cells by glucose, supports the hypothesis that postprandial glucose spikes are important in the early development of both microvascular and macrovascular diseases[19].

The hypoglycemic studies on the aqueous leaf extract of traditional medicinal plant *P. amarus* revealed significant hypoglycemic effect in the glucose loaded as well as STZ induced diabetic rat models. The glucose lowering effect of the plant extract in the present study might be attributed to delayed intestinal glucose absorption and/or increased glucose utilization by the intestine with reference to anaerobic glucose metabolism thereby creating decreased passage of glucose from mucosal side to serosal side of the intestine.

Further studies were designed to understand the intestinal glucose uptake both in the presence and absence of the plant extract. The results obtained in the present *ex vivo* study envisaged that the aqueous leaf extract of *P. amarus* significantly inhibits glucose absorption/transport in the everted rat gut and significantly reduced when compared to control.

The Michelis-Menten constant (Km) of the glucose uptake was calculated for all the experiments. Km is the affinity of the transferring enzyme for the substrate. In the present study it is simulated that Km is the affinity of glucose transporters i.e. GLUT2 and SGLT1 for glucose.

The maximal velocity (V_{max}) is regarded as the glucose uptake rate in the presence as well as in the absence of plant extract. The decrease in the V_{max} in the presence of the aqueous extract of the studied plant extract indicated that the transmembranal glucose transport was significantly decreased. However the Km remained unaltered in the presence as well as in the absence of the extract. This indicates that the aqueous plant extract of *P. amarus* act by bringing a non-competitive type of inhibition of transport of glucose at the level of small intestine. This might be due to the inhibition of glucose transporter proteins (GLUT2 and SGLT1) activity. Previous studies in our lab[6], recorded that the aqueous seed extract of *Trigonella foenum-graecum* (*T. foenum graecum*) inhibited the glucose absorption in rat everted gut sacs and studies elsewhere recorded that the aqueous extracts of *T. foenum graecum* (seed) and *Eugenia jambolana* (seed) decreases the glucose absorption in rat everted gut sacs[20].

Inhibition of intestinal and renal Na^+-glucose co-transporter by naringenin. The aqueous extract of *Momordica charantia* inhibited the glucose transported across the rat everted gut sacs *in-vitro*[4]. *Nigella sativa* inhibits intestinal glucose absorption and improve glucose tolerance in rats[21]. Oral administration of aporphines and secoaporphines had an inhibitory effect on intestinal glucose uptake[22]. The above studies in our lab revealed that the aqueous extract of traditional medicinal plant, *P. amarus* contain phytochemical constituents that are potent to inhibit the glucose uptake across intestinal membrane which might be due to decreased activity of glucose transporter transmembranal proteins in the small intestine of

rat and thereby opening new scope for postprandial glycemic control.

Based on the data obtained in the present study we propose that *P. amarus* aqueous leaf extract possesses hypoglycemic property that inhibits the glucose transport at the site of intestinal brush border membrane in rats.

Conflict of interest statement

The authors report no conflict of interest.

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